

# Serum 25-hydroxyvitamin D and risk of post-menopausal breast cancer—results of a large case–control study

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Various studies suggest that vitamin D may reduce breast cancer risk. Most studies assessed the effects of dietary intake only, although endogenous production is an important source of vitamin D. Therefore, the measurement of serum 25-hydroxyvitamin D [25(OH)D] better indicates overall vitamin D status. To assess the association of 25(OH)D serum concentrations with post-menopausal breast cancer risk, we used a population-based case–control study in Germany, which recruited incident breast cancer patients aged 50–74 between 2002 and 2005. Information on socio-demographic and breast cancer risk factors was collected by personal interview. For this analysis, we included 1394 cases and 1365 controls, matched on year of birth and time of blood collection. Conditional logistic regression was used to calculate odds ratios (ORs) for breast cancer adjusted for potential confounders. Serum 25(OH)D concentration was significantly inversely associated with post-menopausal breast cancer risk. Compared with the lowest category (<30 nM), OR [95% confidence intervals (CI)] for the higher categories of 25(OH)D (30–45, 45–60, 60–75 and ≥75 nM) were 0.57 (0.45–0.73), 0.49 (0.38–0.64), 0.43 (0.32–0.57) and 0.31 (0.24–0.42), respectively ( $P_{\text{trend}} < 0.0001$ ). Analysis using fractional polynomials indicated a non-linear association. The association was stronger in women never using menopausal hormone therapy (HT) compared with past and current users ( $P_{\text{interaction}} < 0.0001$ ). Our findings strongly suggest a protective effect for post-menopausal breast cancer through a better vitamin D supply as characterized by serum 25(OH)D measurement, with a stronger inverse association in women with low serum 25(OH)D concentrations (<50 nM).

## Introduction

Epidemiological and experimental studies suggest an inverse association between vitamin D and cancer of different sites, including breast cancer (1–6). Most epidemiological studies have assessed the effects of dietary intake only, although endogenous production after sun exposure is the main source of vitamin D (6). Both vitamin D from diet and endogenous production is converted via two consecutive hydroxylation steps to 25-hydroxyvitamin D [25(OH)D] in the liver and to the biologically active form of vitamin D 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] in the kidneys. In addition to its essential role in calcium homeostasis and bone metabolism, 1,25(OH)<sub>2</sub>D has a wide range of non-classical actions that include induction of cell differentiation, inhibition of cell growth and regulation of apoptosis in normal and malignant cells, including human breast cancer cells (7–10). 1,25(OH)<sub>2</sub>D exerts its anticarcinogenic actions via the vitamin D

**Abbreviations:** BMI, body mass index; CI, confidence interval; ER, estrogen receptor; HT, hormone therapy; NHS, Nurses' Health Study; OR, odds ratio; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)<sub>2</sub>D, 1,25-dihydroxyvitamin D; PR, progesterone receptor; R-N-K, Rhein–Neckar–Karlsruhe; VDR, vitamin D receptor.

receptor (VDR) modulating the transcription of target genes such as p21, p27, c-fos and c-myc (1).

Although not biologically active, there is overall agreement that 25(OH)D is the appropriate biomarker with which to measure vitamin D status in humans. In contrast to 1,25(OH)<sub>2</sub>D, 25(OH)D is not tightly regulated and better reflects vitamin D stores obtained from both sunlight exposure and ingested vitamin D over longer periods (11–14). Recent studies have demonstrated that conversion to the biologically active form of vitamin D 1,25(OH)<sub>2</sub>D also occurs in extrarenal tissues such as colon, prostate and breast (15–17). In addition, a recent cell study reported expression of CYP27B1, the enzyme that converts 25(OH)D to active 1,25(OH)<sub>2</sub>D, in mammary cells as well as growth inhibition of mammary cells by 25(OH)D, thus linking vitamin D status to breast cancer risk (18).

Data from epidemiologic studies assessing the association between dietary intake of vitamin D and breast cancer risk have been inconclusive (5,19–25). Thus far, only four studies have assessed the relationship between serum vitamin D metabolites [25(OH)D or 1,25(OH)<sub>2</sub>D] and breast cancer risk (2,4,26,27). Of two small hospital-based case–control studies, one found a significantly inverse association for 25(OH)D and breast cancer risk (4), whereas the other study revealed no association for 25(OH)D but an inverse association for 1,25(OH)<sub>2</sub>D (27). A case–control study nested in a voluntary health check up showed no association between 1,25(OH)<sub>2</sub>D and breast cancer risk (26). To date, the largest case–control study including 701 cases nested in the Nurses' Health Study (NHS) (2) reported a non-significant decrease in breast cancer risk both with higher serum 25(OH)D and with 1,25(OH)<sub>2</sub>D concentrations.

To our knowledge, the present study including 1394 cases is by far the largest population-based case–control study assessing the relationship of serum 25(OH)D concentrations and breast cancer risk in post-menopausal women. Because estrogens are known to increase concentrations of vitamin D-binding protein, VDR and serum 25(OH)D (28–34), we additionally examined effect modification by risk factors including hormone-related variables such as menopausal hormone therapy (HT) and number of pregnancies.

## Materials and methods

### Study population and data collection

We used data from a large population-based case–control study (MARIE study, Mamma Carcinoma Risk factor Investigation) carried out in two regions in Germany, the city of Hamburg and the Rhein–Neckar–Karlsruhe (R-N-K) region. The study was approved by the ethics committees of both the University of Heidelberg and the University of Hamburg and conducted in accordance with the Declaration of Helsinki. All study participants gave informed consent. Cases were eligible if they had a histologically confirmed primary invasive or *in situ* breast cancer diagnosed between 01 January 2001 and 30 September 2005 in Hamburg and between 01 August 2002 and 31 July 2005 in the R-N-K region. Further inclusion criteria were age between 50 and 74 and being a resident of one of the study regions. Cases were identified through frequent monitoring of hospital admissions, surgery schedules and pathology records. Clinical and pathological characteristics of the patients were abstracted from hospital and pathology records. Of the 5970 eligible patients who could be contacted, 3919 (65.6%) participated, whereas 2051 (34.4%) declined participation or did not respond to invitation letters.

Two controls per case were randomly selected from lists of residents provided by population registries and frequency matched by year of birth and study region to the cases. Of the 17 093 controls who met the inclusion criteria, 7421 (43.4%) participated, 7521 (44.0%) refused to participate and 2151 (12.6%) did not respond.

All participants were interviewed by trained personnel to obtain information on sociodemographic factors, anthropometric measures, data on lifetime HT exposure, including information on timing and duration, type, dose and brand name of HT, and other potential breast cancer risk factors. An alphabetical list with photographic depictions of >300 HT products prescribed in Germany

over the past 35 years was presented to women as a memory aid recalling their HT history. In addition, participants reported their dietary habits during the past 12 months with a self-administered validated 176 items' food frequency questionnaire (35). Nutrient intake was calculated with the German food composition table BLS II.3 (Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany).

#### Measurement of 25(OH)D

For quantification of 25(OH)D in serum, we used the OCTEIA 25(OH)D enzyme immunoassay (Immunodiagnostic Systems Limited, Boldon, UK). Serum samples were stored in aliquots at  $-80^{\circ}\text{C}$  until measurement. Samples were analyzed in a single batch between November 2006 and January 2007. The coefficient of variation was 3.4% for intraassay determination and 7.6% for interassay determination. Recovery was analyzed by spiking a high with a low serum concentration sample (98.7 and 26.3 nM, respectively) and comparing the observed with the expected values. When spiking the high- and low-concentration samples in a 1:1, 3:1 and 1:3 ratio, recovery was 105, 99 and 96%, respectively. We measured 235 random samples (8.5%) in duplicate. The average absolute deviation from the mean between two duplicates was 2.2%.

#### Data analysis

Women were defined as post-menopausal if they reported their last natural menstrual bleeding at least 12 months before the reference date, a bilateral oophorectomy or cessation of menses due to radiation or chemotherapy. In addition, women  $>55$  years with unclear menopausal status due to hysterectomy or hormone use were also considered post-menopausal (since the 90th percentile for age at menopause for women with natural menopause was 55 years). Women under age 55 with unclear menopausal status and women who were clearly pre-menopausal were excluded from the analysis. A total of 3464 invasive or *in situ* breast cancer cases and 6657 controls, of which 1559 cases and 3008 controls came from the R-N-K region, were classified as post-menopausal. For this analysis, we identified 1400 post-menopausal cases from the R-N-K region for whom we had blood samples (90% of the cases from the study region) and randomly selected 1400 post-menopausal controls from the same study region with matching on time of blood collection in four categories (January–March, April–June, July–September and October–December) and year of birth (continuous). After exclusion of hemolytic serum samples, our final study population consisted of 1394 post-menopausal cases and 1365 post-menopausal controls.

We assessed the association of serum 25(OH)D and post-menopausal breast cancer risk by means of logistic regression with stratification by year of birth (continuous) and time of blood collection (four categories). Odds ratio (OR) estimates and their 95% confidence intervals (CIs) were calculated assessing 25(OH)D concentration both as continuous (per 10 nM increment) and as categorical variable divided into five categories ( $<30$ , 30–45, 45–60, 60–75,  $\geq 75$  nM). This categorization closely corresponds to the classification into quintiles according to the distribution in the control group. We present OR estimates adjusted for age at menopause ( $<47$ , 47–51, 52–55,  $\geq 56$ , unknown), body mass index (BMI) ( $<22.5$ , 22.5–25, 25–30,  $\geq 30$  kg/m<sup>2</sup>), education level (low, middle, high), first-degree family history of breast cancer (yes, no, unknown), history of benign breast disease (yes, no), number of pregnancies ( $\geq 28$ th week) (0, 1, 2,  $\geq 3$ ), age at menarche ( $<12$ , 12–14,  $\geq 15$ ), breastfeeding history (ever, never), total number of mammograms (0, 1–4, 5–9,  $\geq 10$ , unknown), smoking status (never, past, current) and use of menopausal HT (never, past, current). Women reporting HT use for  $\leq 3$  months before the reference date were considered 'never' users and women with  $>3$  months were considered 'ever' users. Among the ever users, women were defined as 'current' users of HT if they reported use within 6 months before the reference date; otherwise they were defined as 'past' users. Adjustment on type of menopause, type of HT, age at first birth, alcohol consumption, contraceptive use, physical activity, dietary intake of calcium and dietary intake of vitamin D did not affect the OR estimates substantially and were therefore not included in the fully adjusted models. Test for linear trend was performed using the median values in each category as an ordinal variable. To further examine dose–response relation and non-linearity of the log OR function for predictive 25(OH)D concentrations, we used the method of fractional polynomials (36). The continuous 25(OH)D variable was entered into the multivariate logistic regression model via a set of defined transformations [ $x^{-2}$ ,  $x^{-1}$ ,  $x^{-0.5}$ ,  $x^{0.5}$ ,  $x^2$ ,  $x^3$  and  $\log(x)$ ], allowing a maximum of two terms (including the untransformed variable) in the model. The function that best fitted the data was selected on the basis of the  $-2 \log$  likelihood of the respective model. We additionally fitted the multivariate logistic regression models with restricted cubic splines for 25(OH)D concentration treated as a continuous variable (37). We specified four knot points at 25(OH)D concentrations of 9.7, 36, 51.5 and 70.7 representing the minimum, the 25th, 50th and 75th percentiles in the control group, respectively. Other knot points were also specified but did not change the shape of the OR function.

We assessed effect modification by testing for multiplicative interaction. We included an interaction term of the continuous variable of interest [25(OH)D] and potential interaction variables in the fully adjusted model and evaluated statistical significance with the likelihood ratio test. Differences in cases and controls were evaluated with the  $\chi^2$  test. All tests were two sided and considered to be statistically significant if  $P \leq 0.05$ . All statistical analyses were carried out using the statistical software SAS 9.1 (SAS Institute, Cary, NC).

## Results

The mean age of cases and controls was 63.6 and 63.5 years, respectively. Characteristics, including sociodemographic variables and main risk factors for breast cancer, of the 1394 post-menopausal cases and 1365 post-menopausal controls are shown in Table I. Cases and controls differed significantly with respect to age at menopause, family history of breast cancer in at least one first-degree relative, benign breast disease history, number of pregnancies, breastfeeding history, age at menarche, number of mammograms and use of menopausal HT (Table I).

Median serum concentration of 25(OH)D was 44.9 and 51.5 nM in cases and controls, respectively ( $P < 0.0001$ ). We found a significant inverse association between serum concentration of 25(OH)D and risk of post-menopausal breast cancer. Compared with the lowest category ( $<30$  nM), the ORs (95% CI) for higher serum concentrations (30–45, 45–60, 60–75 and  $\geq 75$  nM) were 0.57 (0.45–0.73), 0.49 (0.38–0.64), 0.43 (0.32–0.57) and 0.31 (0.24–0.42), respectively ( $P_{\text{trend}} < 0.0001$ ) (Table II). We additionally analyzed 25(OH)D as a continuous variable in the multivariate model and found a significantly reduced risk for post-menopausal breast cancer of 0.88 (0.85–0.91) per 10 nM increment in serum 25(OH)D (Table II). We further examined the shape of the risk function using fractional polynomials and found a non-linear association between the log OR and the 25(OH)D concentration ( $P_{\text{non-linearity}} = 0.0002$ ). The model with the polynomial  $(x + 1)^{-0.5}$  best fitted the data. The resulting function,  $\text{OR}(x) = \exp[10.4 \times (x + 1)^{-0.5}]$ , taking the median value of the reference category from the categorical analysis [24.3 nM 25(OH)D in the controls] as the reference, is displayed in Figure 1. Our data suggest a more pronounced inverse association between 25(OH)D and breast cancer risk in the lower concentration range [ $<50$  nM 25(OH)D] and a flattening of the risk function with increasing 25(OH)D concentrations. There was a good fit between the curve and the OR estimates of the categorical analysis. Using restricted cubic spline regression, the shape of the risk function was similar, but model fit was inferior compared with the model using fractional polynomials.

Following previous reports on the interaction between the estrogen and the vitamin D endocrine system, we evaluated effect modification by use of HT. Median serum 25(OH)D concentrations were higher in women currently using HT compared with past or never users. However, these differences were more prominent in cases than in controls (Table III). In women who reported never having used HT, breast cancer risk reduction per 10 nM increment in 25(OH)D was greater [OR (95% CI) = 0.78 (0.73–0.83)] than in past and current hormone users [OR (95% CI) = 0.86 (0.79–0.94) and 0.96 (0.91–1.02), respectively] (Table III). The interaction between 25(OH)D concentration and use of HT was statistically significant ( $P_{\text{interaction}} < 0.0001$ ) and independent of the type of HT used (estrogen-only or combined estrogen–progestin therapy). The effect modification by HT use also did not differ by estrogen receptor (ER) or progesterone receptor (PR) status of the tumor.

The effect of 25(OH)D on breast cancer risk was also modified by number of pregnancies ( $P_{\text{interaction}} = 0.004$ ). The OR (95% CI) for breast cancer per 10 nM increment in 25(OH)D in women with less than two pregnancies was 0.91 (0.87–0.96), while the association was stronger in women with two [0.86 (0.82–0.92)] and three or more pregnancies [0.81 (0.73–0.89)] (Table IV).

The association between 25(OH)D concentration and breast cancer risk was not significantly modified by age, smoking status, age at menarche, age at menopause, BMI, alcohol, family history of breast

**Table I.** Characteristics and risk factors for post-menopausal breast cancer in cases and matched controls in the study population

Characteristics	Cases (N = 1394)		Controls (N = 1365)		P <sup>a</sup>
	n	%	n	%	
Age at diagnosis/recruitment (years)					
50–54	91	6.5	86	6.3	0.84
55–59	300	21.5	272	19.9	
60–64	447	32.1	457	33.5	
65–69	380	27.3	372	27.3	
≥70	176	12.6	178	13.0	
BMI (kg/m <sup>2</sup> )					
<22.5	544	39.0	536	39.3	0.75
22.5–25	470	33.7	436	32.0	
25–30	322	23.1	333	24.4	
≥30	58	4.2	659	4.3	
Missing values			1		
Educational level					
Low	905	64.9	923	67.6	0.12
Middle	299	21.5	290	21.3	
High	190	13.6	152	11.1	
Age at menopause (years)					
<47	146	10.5	210	15.4	<0.01
47–51	399	28.6	384	28.1	
52–55	243	17.4	196	14.4	
≥56	63	4.5	65	4.8	
Unknown	543	39.0	510	37.3	
First-degree family history of breast cancer					
No	1102	79.1	1118	81.9	0.01
Yes	232	16.6	181	13.3	
Unknown	60	4.3	66	4.8	
Benign breast disease					
No	848	61.0	976	72.8	<0.01
Yes	542	39.0	383	27.2	
Missing values	4		6		
Number of pregnancies (≥28th week)					
0	199	14.3	163	11.9	<0.01
1	377	27.0	311	22.8	
2	514	36.9	555	40.7	
≥3	304	21.8	336	24.6	
Age at menarche (years)					
<12	126	9.0	120	8.8	0.04
12–14	938	67.3	862	63.3	
≥15	330	23.7	380	27.9	
Missing values			3		
Ever breastfeeding					
No	550	39.5	457	33.5	<0.01
Yes	844	60.5	908	66.5	
Number of mammograms in total					
0	192	13.8	175	12.8	<0.01
1–4	590	42.3	720	52.8	
5–9	344	24.7	317	23.2	
≥10	250	17.9	145	10.6	
Unknown number	18	1.3	8	0.6	
Use of HT					
Never	524	38.0	595	44.0	<0.01
Past	288	20.9	346	25.6	
Current (≤6 months)	567	41.1	411	30.4	
Missing values	15		13		
Smoking					
Never	876	62.8	806	59.1	0.05
Past	317	22.8	365	26.7	
Current	201	14.4	194	14.2	
Alcohol consumption (g/day)					
0	247	17.7	222	16.3	0.51
>0–18	977	70.1	983	72.0	
≥19	170	12.2	160	11.7	
Physical activity in quintiles (MET; h/week) <sup>b</sup>					
<145.3	281	20.3	273	20	0.41
145.3–179.3	293	21.1	272	20	
179.4–213.1	303	21.8	273	20	
213.2–257.0	243	17.5	273	20	
≥257.1	268	19.3	272	20	
Missing values	6		2		

**Table I. Continued**

Characteristics	Cases (N = 1394)		Controls (N = 1365)		P <sup>a</sup>
	n	%	n	%	
Hormonal receptor status of the tumor <sup>c</sup>					
ER					
Positive	984	76.4			
Negative	304	23.6			
PR					
Positive	842	65.4			
Negative	445	34.6			
Time of blood collection					
January–March	319	22.9	311	22.8	0.99
April–June	292	21.0	286	21.0	
July–September	402	28.8	392	28.7	
October–December	381	27.3	376	27.5	

<sup>a</sup>χ<sup>2</sup> test.

<sup>b</sup>MET, metabolic equivalents.

<sup>c</sup>Data on ER and PR status were available for 1288 and 1287 invasive tumor cases (*in situ* tumors excluded), respectively.

cancer, breastfeeding history, history of benign breast disease, physical activity or dietary intake of calcium. In addition, no modification by ER or PR status of the tumor or combinations of both was observed. We further examined the association of serum 25(OH)D and breast cancer risk within strata of dietary vitamin D intake. The association remained inverse and no statistical interaction was observed. In addition, dietary intake was not correlated with serum 25(OH)D ( $r = 0.03$ ,  $P = 0.14$ ).

Median difference between time of diagnosis and time of blood collection in cases was 66 days. In a sensitivity analysis, we evaluated potential effects of diagnosis or cancer therapy on circulating 25(OH)D concentration by excluding patients close to diagnosis. Compared with the lowest category (<30 nM), the ORs (95% CI) for the highest category (≥75 nM) were 0.31 (0.24–0.42) for all cases and 0.32 (0.23–0.43) and 0.39 (0.26–0.58), respectively, when excluding cases with <15 days (26% of all cases) and cases with <6 months (63% of all cases) between time of diagnosis and time of blood collection.

## Discussion

In this population-based case–control study, the concentration of serum 25(OH)D was significantly inversely associated with the risk of post-menopausal breast cancer. To date, only three studies have assessed the association of breast cancer risk and 25(OH)D (2,4,27). Our findings support the recently reported results from a case–control study nested in the NHS (2). The authors found an inverse, although not significant, association between breast cancer risk and both 25(OH)D and 1,25(OH)<sub>2</sub>D metabolites restricted to post-menopausal women only. Lowe *et al.* (4) also observed a significant risk reduction of breast cancer in the highest category of 25(OH)D compared with the lowest in predominantly post-menopausal women. In contrast, Janowsky *et al.* (27) reported no association of breast cancer with serum 25(OH)D but with 1,25(OH)<sub>2</sub>D levels. However, the authors included both pre- and post-menopausal women and used hospital-based controls, who may have had less sun exposure than population-based controls. This might explain the conflicting results for 25(OH)D and 1,25(OH)<sub>2</sub>D as less sun exposure has a greater impact on 25(OH)D status than on the more tightly regulated 1,25(OH)<sub>2</sub>D concentration (14). Recently, a case–control study found lower levels of 1,25(OH)<sub>2</sub>D but not 25(OH)D in breast cancer patients (38). However, this study was very small and no risk estimates were reported.

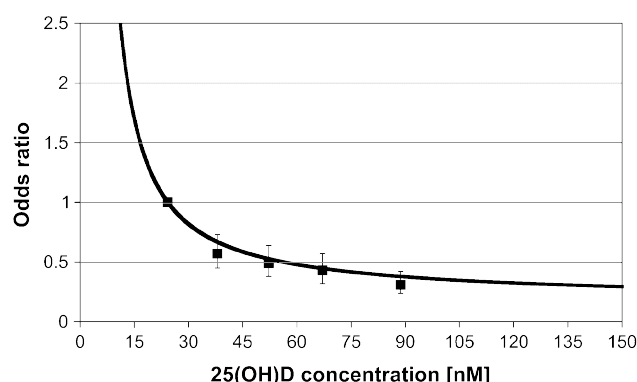
Results regarding the association between vitamin D metabolites and breast cancer risk are not easy to compare because of different metabolite levels in the study populations and reference groups used for calculating relative risks. In our study, median serum 25(OH)D

**Table II.** ORs for post-menopausal breast cancer by serum 25(OH)D concentration

Serum 25(OH)D (nM)	Cases		Controls		Crude model <sup>a</sup> OR (95% CI)	Adjusted model <sup>b</sup> OR (95% CI)
	n	%	n	%		
Categorized						
<30	345	24.8	218	16.0	1	1
30–45	354	25.4	327	23.9	0.65 (0.52–0.82)	0.57 (0.45–0.73)
45–60	300	21.5	308	22.6	0.56 (0.44–0.71)	0.49 (0.38–0.64)
60–75	186	13.3	218	16.0	0.49 (0.37–0.64)	0.43 (0.32–0.57)
≥75	209	15.0	294	21.5	0.39 (0.30–0.50)	0.31 (0.24–0.42)
<i>P</i> <sub>trend</sub>					<0.0001	<0.0001
As continuous variable						
Per 10 nM increment	1394		1365		0.89 (0.87–0.92)	0.88 (0.85–0.91)

<sup>a</sup>Conditional logistic regression stratified by time of blood collection and year of birth.

<sup>b</sup>Conditional logistic regression stratified by time of blood collection and year of birth adjusted for age at menopause, first-degree family history of breast cancer, history of benign breast disease, number of pregnancies (≥28th week), age at menarche, breastfeeding history, total number of mammograms, use of HT, BMI, education level, smoking status; due to missing values, 40 observations were not included in the adjusted model.



**Fig. 1.** ORs for post-menopausal breast cancer by 25(OH)D concentrations using fractional polynomial as dose–response analysis. The resulting function  $OR(x) = \exp[10.4 \times (x + 1)^{-0.5}]$  is displayed, setting the median of the lowest category from the categorical analysis as reference [24.3 nM 25(OH)D]. The solid squares and respective bars represent the ORs and 95% CIs of the categorical analysis (30–45, 45–60, 60–75 and ≥75 nM). For graphical illustration categorical OR estimates were displayed at the median value in the controls of each category, at 24.3 (reference), 38.0, 52.2, 67.1 and 88.7 nM, respectively.

concentrations of 44.9 and 51.5 nM for cases and controls, respectively, were compared with recently published data from the German National Health Survey for women in this age range (39). However, 25(OH)D concentrations were relatively low compared with other studies in the USA and the UK reporting mean concentrations of >80 nM (2,4). We therefore repeated our analysis calculating OR estimates for breast cancer risk comparing women with concentrations >100 nM 25(OH)D to women with concentrations <50 nM 25(OH)D, which is comparable with the categorization in the NHS (2). The observed inverse association was considerably stronger in our study population compared with that found in the NHS [OR (95% CI) = 0.44 (0.30–0.65) and 0.73 (0.49–1.07), respectively].

There is ample evidence from cellular and animal studies linking 25(OH)D to breast cancer. This includes the known anticarcinogenic effects of vitamin D with regard to apoptosis, cell differentiation and proliferation, growth inhibition of human mammary epithelial cells by both 1,25(OH)<sub>2</sub>D and 25(OH)D, the presence and expression of the VDR and CYP27B1, the enzyme that converts 25(OH)D to active

1,25(OH)<sub>2</sub>D, in mammary cells, and the uptake of the vitamin D-binding protein–25(OH)D complex in mammary cells *in vitro* (40). However, little is known about the delivery of 25(OH)D to mammary cells and therefore further investigations into the mechanisms of delivery, uptake and action of 25(OH)D in mammary cells are needed.

Our findings of a non-linear relationship between 25(OH)D concentrations and the log OR for breast cancer using fractional polynomials are of special interest. In our study population, the protective effect was strongest in women with low 25(OH)D concentrations (<50 nM) and was reduced substantially with higher 25(OH)D concentration. These are important public health findings for women in low 25(OH)D concentration ranges and indicate subgroups where vitamin D supplementation may be most relevant for breast cancer prevention. Due to the low 25(OH)D concentrations in our study population, we were able to provide important additional information to further understand the dose–response relationship of vitamin D and breast cancer risk.

We found a statistically significant modification of the association of breast cancer risk and serum 25(OH)D concentrations with a stronger effect in women never having used HT as compared with HT users. Accordingly, results from the NHS showed some evidence for a stronger effect in non-HT users, although power was low for their comparisons (2). In women using HT, taking oral contraceptives or with increased endogenous estrogen levels, higher concentrations of vitamin D metabolites have been reported (32,33,41,42). The underlying mechanisms include the estrogen-induced activation of renal 1 $\alpha$ -hydroxylase, inhibition of 24-vitamin D hydroxylase and the up-regulation of the VDR resulting in increased serum concentrations and activity of 25(OH)D and 1,25(OH)<sub>2</sub>D (33,41,43,44). We also found higher 25(OH)D serum concentrations in women currently using HT compared with past or never users, and this difference was not attributable to differences in age, BMI, physical activity or other known breast cancer risk factors. Serum 25(OH)D concentrations in women never using HT were significantly lower in cases than in controls ( $P < 0.0001$ ), whereas no significant case–control differences were observed in women currently using HT. We hypothesize that HT may induce an increase in 25(OH)D only below a certain threshold level of 25(OH)D (median: 37.3 and 47.6 nM for cases and controls never using HT, respectively), resulting in differential effects in cases and controls in our study population and in effect modification by HT on the association of 25(OH)D with breast cancer risk. In line with this hypothesis, use of HT was significantly associated with 25(OH)D using a linear regression model in women with low serum 25(OH)D concentrations (<50 nM,  $\beta = 2.2$ ,  $P = 0.004$ ) but not in women with high serum concentrations (>50 nM,  $\beta = -2.2$ ,  $P = 0.21$ ). To our knowledge, there is, however, no data biologically supporting this threshold hypothesis.

We also found a stronger effect of 25(OH)D on breast cancer risk in women with increasing number of pregnancies. Parity is known to be associated with changes in sex hormone level resulting in a potential lower lifetime estrogen exposure (45,46). Cell studies on the interaction of estrogens, anti-estrogens and 1,25(OH)<sub>2</sub>D in tumor cell lines have been conducted. In cell studies, 1,25(OH)<sub>2</sub>D has been shown to be considerably more anticarcinogenic in combination with anti-estrogens (47,48). In addition, in the presence of estradiol, 1,25(OH)<sub>2</sub>D has been shown to stimulate proliferation at low doses and inhibit proliferation at higher doses, whereas in a non-estrogen environment, only anti-proliferative effects have been observed (48). Therefore, both, the interaction of 25(OH)D with use of HT and with the number of pregnancies, could be explained by interactions with the estrogen metabolism, i.e. a lower lifetime exposure to estrogens resulting in a potential stronger anticarcinogenic effect of vitamin D. However, the complex regulation of vitamin D in the human body and its interactions with the estrogen metabolism in breast cancer cells deserve further investigation.

We further analyzed whether the effect of vitamin D on breast cancer differed by receptor status of the tumor and did not find differential effects. Recent data from the NHS suggest an inverse association for ER–/PR–, but not for ER+/PR+ and ER+/PR– tumors,



**Table III.** ORs<sup>a</sup> for post-menopausal breast cancer by serum 25(OH)D concentration by use of HT

Serum 25(OH)D (nM)	Never HT use		Past HT use		Current HT use	
	Ca/Co	OR (95% CI)	Ca/Co	OR (95% CI)	Ca/Co	OR (95% CI)
Median	37.3/47.6		45.8/53.4		51.5/54.0	
Categorized	<i>n</i>		<i>n</i>		<i>n</i>	
<30	193/125	1	58/46	1	89/44	1
30–45	130/149	0.51 (0.36–0.74)	82/71	0.62 (0.33–1.14)	140/105	0.57 (0.35–0.92)
45–60	101/123	0.45 (0.30–0.68)	59/83	0.36 (0.19–0.68)	136/98	0.61 (0.37–1.01)
60–75	56/81	0.30 (0.19–0.49)	48/72	0.35 (0.18–0.69)	80/63	0.60 (0.34–1.05)
≥75	44/117	0.18 (0.11–0.30)	41/74	0.25 (0.12–0.51)	122/101	0.49 (0.29–0.83)
<i>P</i> <sub>trend</sub>		<0.0001		0.0001		0.05
As continuous variable <sup>b</sup>						
Per 10 nM increment	524/595	0.78 (0.73–0.83)	288/346	0.86 (0.79–0.94)	567/411	0.96 (0.91–1.02)

Ca, cases; Co, controls.

<sup>a</sup>Conditional logistic regression stratified by time of blood collection and year of birth adjusted for age at menopause, first-degree family history of breast cancer, history of benign breast disease, number of pregnancies (≥28th week), age at menarche, breastfeeding history, total number of mammograms, BMI, education level, smoking status; due to missing values, 40 observations were not included in the model.

<sup>b</sup>*P*<sub>interaction</sub> < 0.0001.

**Table IV.** ORs<sup>a</sup> for post-menopausal breast cancer by serum 25(OH)D by number of pregnancies

Serum 25(OH)D (nM)	0–1 pregnancies		2 pregnancies		≥3 pregnancies	
	Ca/Co	OR (95% CI)	Ca/Co	OR (95% CI)	Ca/Co	OR (95% CI)
Median	45.9/50.1		45.2/53.6		42.3/49.7	
Categorized	<i>n</i>		<i>n</i>		<i>n</i>	
<30	139/76	1	119/82	1	87/60	1
30–45	136/119	0.42 (0.27–0.65)	134/123	0.72 (0.47–1.08)	84/85	0.51 (0.29–0.88)
45–60	122/107	0.40 (0.26–0.63)	104/127	0.50 (0.33–0.78)	74/74	0.46 (0.25–0.82)
60–75	75/70	0.39 (0.24–0.65)	76/88	0.51 (0.31–0.82)	35/60	0.28 (0.14–0.55)
≥75	104/102	0.36 (0.22–0.58)	81/135	0.31 (0.19–0.49)	24/57	0.23 (0.11–0.47)
<i>P</i> <sub>trend</sub>		0.0006		<0.0001		<0.0001
As continuous variable <sup>b</sup>						
Per 10 nM increment	576/474	0.91 (0.87–0.96)	514/555	0.86 (0.82–0.92)	304/336	0.81(0.73–0.89)

Ca, cases; Co, controls.

<sup>a</sup>Conditional logistic regression stratified by time of blood collection and year of birth adjusted for age at menopause, first-degree family history of breast cancer, history of benign breast disease, age at menarche, breastfeeding history, total number of mammograms, use of HT, BMI, education level, smoking status; due to missing values, 40 observations were not included in the model.

<sup>b</sup>*P*<sub>interaction</sub> = 0.004.

although power was low for their analysis (2). Because the effect of HT on breast cancer is known to be stronger in receptor-positive tumors (49,50), we additionally analyzed potential differences in the modification of the breast cancer–25(OH)D association by hormone use in ER+ or PR+ tumors only. No evidence was found for a stronger effect modification in ER+ or PR+ tumors.

With regard to the contribution of dietary vitamin D to serum 25(OH)D, few foods naturally contain vitamin D (i.e. fish and eggs) and fortification of food with vitamin D as well as high fish consumption is not common in Germany. Therefore, a low contribution of dietary vitamin D to the serum 25(OH)D concentration in our study population seems plausible.

Our study may have methodological limitations due to the retrospective case–control design. We are aware that a cancer diagnosis may change dietary or behavioral habits (less dietary intake of vitamin D, less sun exposure), which may influence 25(OH)D concentrations. Nevertheless, short-term changes in diet or behavioral habits are less likely to influence serum concentration because the half-life of 25(OH)D is rather long (2–3 weeks), the correlation between dietary vitamin D and serum 25(OH)D in this study was low ( $r = 0.03$ ) and plasma levels of 25(OH)D are fairly consistent over time (51). Although a notable change in 25(OH)D concentration after chemotherapeutic treatment was not observed in two studies (52,53), cancer therapy following diagnosis may affect circulating 25(OH)D concentration. However,

the sensitivity analysis excluding patients <6 months between diagnosis and blood collection showed no notable change in the risk estimates.

Selection bias due to the participation rate of cases and controls is also unlikely to have biased our results in terms of 25(OH)D status. Information collected via short questionnaire from subjects who refused participation indicated that the study participants were better educated and reported hormone use more frequently than non-participants. However, these differences between study participants and non-participants answering the short questionnaire only were similarly observed both for cases and controls and therefore unlikely to have biased our results. Unfortunately, no information on 25(OH)D status-, diet- or vitamin D-related variables like outdoor activity was available for the non-participants with short questionnaire information.

Finally, we address the concern of possible measurement errors with respect to 25(OH)D status. Several reports emphasize the measurement variation introduced by different assays and different laboratories (14,54,55). Nevertheless, in the international Vitamin D Quality Assessment Scheme, which monitors the performance of vitamin D assays, the IDS enzyme immunoassay used in our study gave comparable results to the gold standard high-performance liquid chromatography method (56). In addition, intra- and interassay variation in 25(OH)D measurement as well as the average absolute deviation from the mean between two duplicate samples were very low in our analysis, reassuring the validity of the biomarker measurement.

In summary, we found a significant inverse association between serum 25(OH)D concentration and post-menopausal breast cancer risk. In addition, the relationship was non-linear, suggesting a stronger effect in women with low 25(OH)D concentrations as compared with women with higher concentrations. Use of HT and number of pregnancies were identified as modifiers of the association between serum 25(OH)D and breast cancer risk. The association was stronger in women who had never used HT compared with women with past or current use and in women with increasing number of pregnancies; however, these findings need confirmation in further studies. Our findings strongly suggest a protective effect for post-menopausal breast cancer risk through a better vitamin D supply as characterized by measurement of serum 25(OH)D.

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## References

- Colston,K.W. *et al.* (2002) Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer. *Endocr. Relat. Cancer*, **9**, 45–59.
- Bertone-Johnson,E.R. *et al.* (2005) Plasma 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D and risk of breast cancer. *Cancer Epidemiol. Biomarkers Prev.*, **14**, 1991–1997.
- Welsh,J. (2004) Vitamin D and breast cancer: insights from animal models. *Am. J. Clin. Nutr.*, **80**, 1721S–1724S.
- Lowe,L.C. *et al.* (2005) Plasma 25-hydroxy vitamin D concentrations, vitamin D receptor genotype and breast cancer risk in a UK Caucasian population. *Eur. J. Cancer*, **41**, 1164–1169.
- Shin,M.H. *et al.* (2002) Intake of dairy products, calcium, and vitamin D and risk of breast cancer. *J. Natl Cancer Inst.*, **94**, 1301–1311.
- Hansen,C.M. *et al.* (2001) Vitamin D and cancer: effects of 1,25(OH)2D3 and its analogs on growth control and tumorigenesis. *Front. Biosci.*, **6**, D820–D848.
- Eisman,J.A. *et al.* (1980) Normal and malignant breast tissue is a target organ for 1,25-(OH)2 vitamin D3. *Clin. Endocrinol. (Oxf)*, **13**, 267–272.
- Saez,S. *et al.* (1993) William L. McGuire Memorial Symposium. 1,25(OH)2D3 modulation of mammary tumor cell growth *in vitro* and *in vivo*. *Breast Cancer Res. Treat.*, **27**, 69–81.
- Jensen,S.S. *et al.* (2001) Inhibitory effects of 1 $\alpha$ ,25-dihydroxyvitamin D(3) on the G(1)-S phase-controlling machinery. *Mol. Endocrinol.*, **15**, 1370–1380.
- Trump,D.L. *et al.* (2004) Anti-tumor activity of calcitriol: pre-clinical and clinical studies. *J. Steroid Biochem. Mol. Biol.*, **89–90**, 519–526.
- Clemens,T.L. *et al.* (1986) Serum vitamin D2 and vitamin D3 metabolite concentrations and absorption of vitamin D2 in elderly subjects. *J. Clin. Endocrinol. Metab.*, **63**, 656–660.
- Reichel,H. *et al.* (1989) The role of the vitamin D endocrine system in health and disease. *N. Engl. J. Med.*, **320**, 980–991.
- Haddad,J.G., Jr *et al.* (1976) Acute administration of 25-hydroxycholecalciferol in man. *J. Clin. Endocrinol. Metab.*, **42**, 284–290.
- Zerwekh,J.E. (2004) The measurement of vitamin D: analytical aspects. *Ann. Clin. Biochem.*, **41**, 272–281.
- Friedrich,M. *et al.* (2003) Analysis of the vitamin D system in cervical carcinomas, breast cancer and ovarian cancer. *Recent Results Cancer Res.*, **164**, 239–246.
- Welsh,J.E. *et al.* (2003) Vitamin D-3 receptor as a target for breast cancer prevention. *J. Nutr.*, **133**, 2425S–2433S.
- Zehnder,D. *et al.* (2001) Extrarenal expression of 25-hydroxyvitamin D(3)-1 $\alpha$ -hydroxylase. *J. Clin. Endocrinol. Metab.*, **86**, 888–894.
- Kemmis,C.M. *et al.* (2006) Human mammary epithelial cells express CYP27B1 and are growth inhibited by 25-hydroxyvitamin D-3, the major circulating form of vitamin D-3. *J. Nutr.*, **136**, 887–892.
- McCullough,M.L. *et al.* (2005) Dairy, calcium, and vitamin D intake and postmenopausal breast cancer risk in the Cancer Prevention Study II Nutrition Cohort. *Cancer Epidemiol. Biomarkers Prev.*, **14**, 2898–2904.
- John,E.M. *et al.* (1999) Vitamin D and breast cancer risk: the NHANES I Epidemiologic follow-up study, 1971–1975 to 1992. National Health and Nutrition Examination Survey. *Cancer Epidemiol. Biomarkers Prev.*, **8**, 399–406.
- Witte,J.S. *et al.* (1997) Diet and premenopausal bilateral breast cancer: a case-control study. *Breast Cancer Res. Treat.*, **42**, 243–251.
- Potischman,N. *et al.* (1999) Intake of food groups and associated micronutrients in relation to risk of early-stage breast cancer. *Int. J. Cancer*, **82**, 315–321.
- Levi,F. *et al.* (2001) Dietary intake of selected micronutrients and breast-cancer risk. *Int. J. Cancer*, **91**, 260–263.
- Simard,A. *et al.* (1991) Vitamin D deficiency and cancer of the breast: an unprovocative ecological hypothesis. *Can. J. Public Health*, **82**, 300–303.
- Abbas,S. *et al.* (2007) Dietary vitamin D and calcium intake and premenopausal breast cancer risk in a German case-control study. *Nutr. Cancer*, **59**, 54–61.
- Hiatt,R.A. *et al.* (1998) Prediagnostic serum vitamin D and breast cancer. *J. Natl Cancer Inst.*, **90**, 461–463.
- Janowsky,E.C. *et al.* (1999) Association between low levels of 1,25-dihydroxyvitamin D and breast cancer risk. *Public Health Nutr.*, **2**, 283–291.
- van Hoof,H.J. *et al.* (2001) Relationship between free and total 1,25-dihydroxyvitamin D in conditions of modified binding. *Eur. J. Endocrinol.*, **144**, 391–396.
- Lips,P. (2001) Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. *Endocr. Rev.*, **22**, 477–501.
- Sowers,M.R. *et al.* (1986) Parameters related to 25-OH-D levels in a population-based study of women. *Am. J. Clin. Nutr.*, **43**, 621–628.
- Nesby-O'Dell,S. *et al.* (2002) Hypovitaminosis D prevalence and determinants among African American and white women of reproductive age: Third National Health and Nutrition Examination Survey, 1988–1994. *Am. J. Clin. Nutr.*, **76**, 187–192.
- Harris,S.S. *et al.* (1998) The association of oral contraceptive use with plasma 25-hydroxyvitamin D levels. *J. Am. Coll. Nutr.*, **17**, 282–284.
- Aarskog,D. *et al.* (1983) Effect of estrogen on vitamin D metabolism in tall girls. *J. Clin. Endocrinol. Metab.*, **57**, 1155–1158.
- Schwartz,B. *et al.* (2000) Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon. *Mol. Cell. Biochem.*, **203**, 87–93.
- Bohlscheid-Thomas,S. *et al.* (1997) Reproducibility and relative validity of energy and macronutrient intake of a food frequency questionnaire developed for the German part of the EPIC project. European Prospective Investigation into Cancer and Nutrition. *Int. J. Epidemiol.*, **26** ((Suppl. 1)), S71–S81.
- Royston,P. *et al.* (1999) The use of fractional polynomials to model continuous risk variables in epidemiology. *Int. J. Epidemiol.*, **28**, 964–974.
- Heinzel,H. *et al.* (1997) Gaining more flexibility in Cox proportional hazards regression models with cubic spline functions. *Comput. Methods Programs Biomed.*, **54**, 201–208.
- de Lyra,E.C. *et al.* (2006) 25(OH)D3 and 1,25(OH)2D3 serum concentration and breast tissue expression of 1 $\alpha$ -hydroxylase, 24-hydroxylase and Vitamin D receptor in women with and without breast cancer. *J. Steroid Biochem. Mol. Biol.*, **100**, 184–192.
- Hintzpeter,B. *et al.* (2007) Vitamin D status and health correlates among German adults. *Eur. J. Clin. Nutr.*
- Welsh,J. (2007) Vitamin D and prevention of breast cancer. *Acta Pharmacol. Sin.*, **28**, 1373–1382.
- Gallagher,J.C. *et al.* (1980) Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. *J. Clin. Endocrinol. Metab.*, **51**, 1359–1364.
- Peters,U. *et al.* (2004) Circulating vitamin D metabolites, polymorphism in vitamin D receptor, and colorectal adenoma risk. *Cancer Epidemiol. Biomarkers Prev.*, **13**, 546–552.
- Buchanan,J.R. *et al.* (1986) The effect of endogenous estrogen fluctuation on metabolism of 25-hydroxyvitamin D. *Calcif. Tissue Int.*, **39**, 139–144.
- Liel,Y. *et al.* (1992) Evidence that estrogens modulate activity and increase the number of 1,25-dihydroxyvitamin D receptors in osteoblast-like cells (ROS 17/2.8). *Endocrinology*, **130**, 2597–2601.
- Bernstein,L. (2002) Epidemiology of endocrine-related risk factors for breast cancer. *J. Mammary Gland Biol. Neoplasia*, **7**, 3–15.

46. Bernstein, L. *et al.* (1985) Estrogen and sex hormone-binding globulin levels in nulliparous and parous women. *J. Natl Cancer Inst.*, **74**, 741–745.
47. Vink-van Wijngaarden, T. *et al.* (1994) Inhibition of breast cancer cell growth by combined treatment with vitamin D3 analogues and tamoxifen. *Cancer Res.*, **54**, 5711–5717.
48. Love-Schimenti, C.D. *et al.* (1996) Antiestrogen potentiation of antiproliferative effects of vitamin D3 analogues in breast cancer cells. *Cancer Res.*, **56**, 2789–2794.
49. Chen, W.Y. *et al.* (2004) Association of hormone replacement therapy to estrogen and progesterone receptor status in invasive breast carcinoma. *Cancer*, **101**, 1490–1500.
50. Ursin, G. *et al.* (2002) Does menopausal hormone replacement therapy interact with known factors to increase risk of breast cancer? *J. Clin. Oncol.*, **20**, 699–706.
51. Feskanich, D. *et al.* (2004) Plasma vitamin D metabolites and risk of colorectal cancer in women. *Cancer Epidemiol. Biomarkers Prev.*, **13**, 1502–1508.
52. Kailajarvi, M.E. *et al.* (2004) Serum bone markers in breast cancer patients during 5-fluorouracil, epirubicin and cyclophosphamide (FEC) therapy. *Anticancer Res.*, **24**, 1271–1274.
53. Gao, Y. *et al.* (1993) The effects of chemotherapy including cisplatin on vitamin D metabolism. *Endocr. J.*, **40**, 737–742.
54. Binkley, N. *et al.* (2004) Assay variation confounds the diagnosis of hypovitaminosis D: a call for standardization. *J. Clin. Endocrinol. Metab.*, **89**, 3152–3157.
55. Hollis, B.W. (2004) Editorial: the determination of circulating 25-hydroxyvitamin D: no easy task. *J. Clin. Endocrinol. Metab.*, **89**, 3149–3151.
56. Carter, G.D. *et al.* (2004) How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme. *Clin. Chem.*, **50**, 2195–2197.

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